

# Neurexins Physically and Functionally Interact with GABA<sub>A</sub> Receptors

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DOI 10.1016/j.neuron.2010.04.008

## SUMMARY

Neurexins are presynaptic cell-adhesion molecules that form *trans*-synaptic complexes with postsynaptic neuroligins. When overexpressed in nonneuronal cells, neurexins induce formation of postsynaptic specializations in cocultured neurons, suggesting that neurexins are synaptogenic. However, we find that when overexpressed in neurons, neurexins do not increase synapse density, but instead selectively suppressed GABAergic synaptic transmission without decreasing GABAergic synapse numbers. This suppression was mediated by all subtypes of neurexins tested, in a cell-autonomous and neuroligin-independent manner. Strikingly, addition of recombinant neurexin to cultured neurons at submicromolar concentrations induced the same suppression of GABAergic synaptic transmission as neurexin overexpression. Moreover, experiments with native brain proteins and purified recombinant proteins revealed that neurexins directly and stoichiometrically bind to GABA<sub>A</sub> receptors, suggesting that they decrease GABAergic synaptic responses by interacting with GABA<sub>A</sub> receptors. Our findings suggest that besides their other well-documented interactions, presynaptic neurexins directly act on postsynaptic GABA<sub>A</sub> receptors, which may contribute to regulate the excitatory/inhibitory balance in brain.

## INTRODUCTION

Neurexins are receptors for the presynaptic neurotoxin  $\alpha$ -latrotoxin (Ushkaryov et al., 1992; Ushkaryov and Südhof, 1993) and

are thought to function as presynaptic cell-adhesion molecules by binding to postsynaptic neuroligins and leucine-rich repeat transmembrane proteins (LRRTMs) (Ichtchenko et al., 1995; Nguyen and Südhof, 1997; Irie et al., 1997; Scheiffele et al., 2000; Ko et al., 2009a; de Wit et al., 2009). In vertebrates, neurexins and neuroligins are synthesized throughout the brain in all excitatory and inhibitory neurons (Ullrich et al., 1995; Ichtchenko et al., 1995, 1996). Neurexins and neuroligins are evolutionarily conserved, indicating that they perform a basic synaptic function (Tabuchi and Südhof, 2002; Li et al., 2007; Zeng et al., 2007; Biswas et al., 2008). Moreover, neurexins belong to a larger protein family that includes neurexin-like proteins such as CASPR/CNTNAPs in vertebrates (Peles et al., 1997), neurexin-IV in *Drosophila* (Baumgartner et al., 1996), and Bam-2 in *C. elegans* (Colavita and Tessier-Lavigne, 2003). All of these molecules function as cell-adhesion and signaling proteins, and contain typical neurexin domains (laminin-neurexin-sex hormone-binding globulin [LNS] domains with interspersed epidermal growth factor [EGF]-like domains), but are otherwise quite different (Missler et al., 1998a). In addition to neuroligins and LRRTMs, neurexins bind to dystroglycan (Sugita et al., 2001) and to neurexophilins (Petrenko et al., 1996; Missler et al., 1998b), but these interactions have not been studied in detail.

Vertebrate genomes contain three neurexin genes, each of which expresses larger  $\alpha$ - and smaller  $\beta$ -neurexins from distinct promoters (Rowen et al., 2002; Tabuchi and Südhof, 2002). Furthermore, extensive alternative splicing of the primary transcripts of all  $\alpha$ - and  $\beta$ -neurexins additionally diversifies neurexins, resulting in potentially thousands of evolutionarily conserved isoforms that are differentially expressed in brain (Ushkaryov et al., 1994; Ullrich et al., 1995; Patzke and Ernsberger, 2000; Zeng et al., 2006; Rissone et al., 2007; Rozic-Kotliroff and Zisapel, 2007). Although affinity of neurexins for neuroligins depends on their alternative splicing (Ichtchenko et al., 1995, 1996; Boucard et al., 2005; Chih et al., 2006; Comoletti et al., 2006), the general significance of neurexin alternative splicing remains unknown.

Endogenous neurexins are largely presynaptic, as evidenced by their role as  $\alpha$ -latrotoxin receptors (Ushkaryov et al., 1992; Sugita et al., 1999), their immunocytochemical localization in brain (Ushkaryov et al., 1992), and their accumulation and functional requirement in presynaptic nerve terminals in artificial synapses (Chubykin et al., 2005; Ko et al., 2009b). In mice, deletion of  $\alpha$ -neurexins causes a presynaptic phenotype that includes a major decrease in action-potential-evoked neurotransmitter release, and a massive impairment in  $\text{Ca}^{2+}$ -channel function (Missler et al., 2003; Zhang et al., 2005). In *Drosophila*, mutation of the single neurexin gene (which encodes an  $\alpha$ -neurexin; Tabuchi and Südhof, 2002) also causes an ultrastructural and functional disorganization of neuromuscular junctions (Li et al., 2007). Consistent with a presynaptic localization and suggestive of a synaptogenic function, overexpressed neurexins in nonneuronal cells induce formation of postsynaptic specializations in co-cultured neurons (Graf et al., 2004; Nam and Chen, 2005). However, neurexins may also have postsynaptic effects because  $\alpha$ -neurexin KO mice display a relative loss of postsynaptic NMDA-receptor activity (Kattenstroth et al., 2004), and neurexins may partly localize postsynaptically (Taniguchi et al., 2007).

Viewed together, the existing data thus suggest that neurexins function as presynaptic cell-adhesion molecules with essential functions in all organisms tested. However, no clear-cut view of the exact nature of these functions has emerged. Whereas the genetic data in mice and flies argue for a role in synapse assembly and function, but not in synaptogenesis (Missler et al., 2003; Li et al., 2007), the induction of postsynaptic specializations by transfected neurexins indicates a synaptogenic function (Graf et al., 2004; Nam and Chen, 2005). Because the effects of neurexin overexpression in neurons have not been examined, it is unclear whether neurexins generally trigger formation of postsynaptic specializations. Moreover, a flurry of recent human genetics studies suggested that changes in the neurexin-1 $\alpha$  gene are associated with autism (Szatmari et al., 2007; Kim et al., 2008; Yan et al., 2008; Marshall et al., 2008; Zahir et al., 2008) and/or schizophrenia (Kirov et al., 2008; Rujescu et al., 2009; Walsh et al., 2008), while changes in the neurexin-3 $\alpha$  gene are linked to addiction (Hishimoto et al., 2007; Lachman et al., 2007; reviewed in Südhof, 2008). Here, we show that neurexins unexpectedly interact with GABA<sub>A</sub> receptors, and that overexpression of neurexins in neurons is not synaptogenic, but selectively inhibits GABAergic synaptic responses by a neuroligin-independent mechanism. These data suggest that neurexins may organize synapses not only via *trans*-synaptic interactions with postsynaptic cell-adhesion molecules, but also with neurotransmitter receptors.

## RESULTS

### Neurexin-2 $\beta$ Downregulates GABAergic Synaptic Transmission

When neuroligins or neurexins are overexpressed in nonneuronal cells that are cocultured with neurons, neuroligins induce formation of presynaptic specializations in the cocultured neurons, while neurexins induce formation of postsynaptic specializations (Scheiffele et al., 2000; Graf et al., 2004; Chubykin et al., 2005; Nam and Chen, 2005). Moreover, overexpression of neuroligins

in neurons increases synapse numbers (Chih et al., 2004; Boucard et al., 2005; Zhang et al., 2009). We therefore hypothesized that overexpression of neurexins in neurons should also increase synapse numbers.

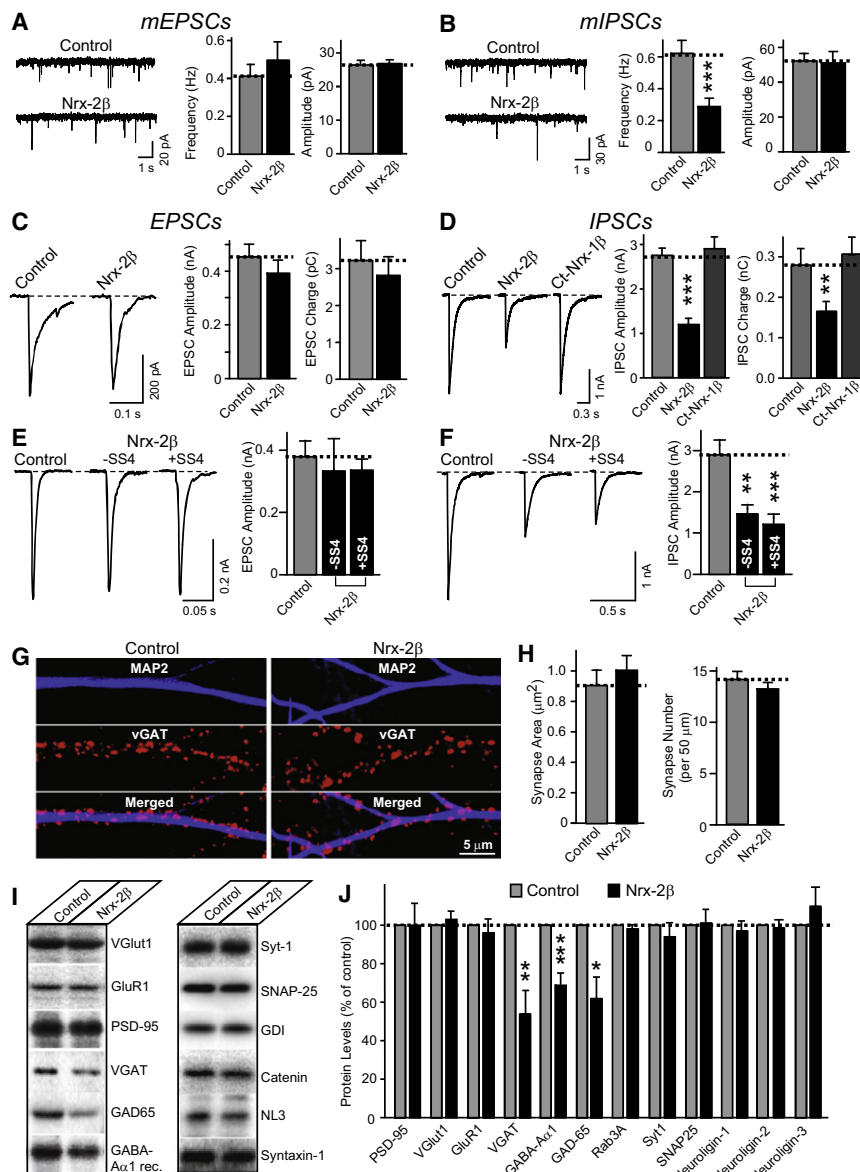
We infected cultured neurons with lentiviruses expressing enhanced green fluorescent protein (EGFP) alone or together with neurexin-2 $\beta$ , such that virtually all neurons are infected. We then measured in these neurons spontaneous miniature excitatory and inhibitory postsynaptic currents (mEPSCs and mIPSCs, respectively). Neurexin-2 $\beta$  caused no change in mEPSCs, but surprisingly induced an  $\sim 2$ -fold decrease in mIPSC frequency, without a significant change in mIPSC amplitude (Figures 1A, 1B, S1A, and S1B). EGFP alone had no effect. We next recorded evoked synaptic responses (ESPCs and IPSCs), and detected a similar selective  $\sim 2$ -fold decrease in IPSC amplitude and charge, again without a significant change in EPSCs (Figures 1C and 1D). This selective impairment in inhibitory GABAergic synaptic transmission by neurexin-2 $\beta$  was independent of alternative splicing at splice site 4 (Figures 1E and 1F).

Using immunocytochemistry, we found that neurexin-2 $\beta$  had no significant effect on the number or size of inhibitory synapses (Figures 1G and 1H). Protein quantitations showed that neurexin-2 $\beta$  caused a small but significant decrease in the levels of pre- and postsynaptic proteins specific for GABAergic synapses, without changing their mRNA levels (Figures 1I, 1J, and S1C). Thus, contrary to expectations, increasing neuronal neurexin-2 $\beta$  expression decreased the function of GABAergic but not glutamatergic synapses, without altering synapse numbers.

### Neurexins Decrease GABAergic Responses in Transfected Neurons

We next transfected neurons with neurexin-2 $\beta$  or a control plasmid, such that only a small percentage of neurons express neurexin-2 $\beta$ , and recorded from the transfected neurons that were identified via cotransfected cyan fluorescent protein (CFP). Similar to lentiviral delivery of neurexin-2 $\beta$ , transfection of neurexin-2 $\beta$  caused a selective  $>2$ -fold decrease in mIPSC frequency (Figures 2A and S2A) and in evoked IPSC amplitude, without altering EPSCs (Figures 2B–2E). To ensure specificity of the observed effect, we measured IPSCs in neighboring transfected and nontransfected neurons on the same coverslip (Figure 2B). Only transfected neurons expressing neurexin-2 $\beta$  exhibited depressed GABAergic synaptic responses. The neurexin-2 $\beta$  induced decrease in IPSCs was not alleviated when neurotransmitter release was increased by elevating the extracellular  $\text{Ca}^{2+}$  concentration (Figures 2C and 2D). Moreover, IPSCs induced by hypertonic sucrose (which induces exocytosis of all readily releasable vesicles) were also significantly decreased by transfected neurexin-2 $\beta$  (Figure 2F).

Similar to panneuronal lentiviral expression of neurexin-2 $\beta$ , transfection of neurexin-2 $\beta$  did not significantly change the density of excitatory or inhibitory synapses (Figures S2B–S2E). Immunocytochemically, no change in the postsynaptic neuroligin signal was observed, whereas we detected a small but significant decrease in the synaptic signal for GABA-A $\alpha 1$  receptors and GAD65 (Figure 2G), consistent with the decrease in GABAergic synaptic protein levels after lentiviral expression of neurexin-2 $\beta$  (Figures 1I and 1J).



**Figure 1. Lentivirally Expressed Neurexin-2 $\beta$  Decreases GABAergic but Not Glutamatergic Synaptic Strength**

All data are from cultured hippocampal neurons infected with lentivirus expressing EGFP only (control), or coexpressing EGFP with neurexin-2 $\beta$  lacking an insert in splice site 4 (Nr2 $\beta$ ), or other versions of neurexin-2 $\beta$  as specified.

(A and B) Representative traces (left) and summary graphs of the frequency (center) and amplitudes (right) of mEPSCs (A) or mIPSCs (B) recorded in 1  $\mu$ M tetrodotoxin (TTX) and 0.1 mM picrotoxin (A) or 10  $\mu$ M CNQX (B) (mEPSCs: control: n = 25 cells/3 cultures; Nr2 $\beta$ : n = 26/3; mIPSCs: control: n = 29/3; Nr2 $\beta$ : n = 27/3).

(C and D) Representative traces (left) and summary graphs of the amplitude (center) and synaptic charge transfer (right, integrated over 0.2 s) of action-potential-evoked EPSCs recorded in 0.1 mM picrotoxin (C) or IPSCs recorded in 10  $\mu$ M CNQX (D; N-terminally truncated neurexin-1 $\beta$  [Ct-Nrx-1 $\beta$ ] was included as additional negative control) (EPSCs: control: n = 54/8; Nr2 $\beta$ : n = 59/8; IPSCs: control: n = 12/3; Nr2 $\beta$ : n = 13/3; Ct-Nrx-1 $\beta$ : n = 15/3).

(E and F) Representative traces (left) and summary graphs of the amplitudes (right) of EPSCs (E) and IPSCs (F) in neurons infected with control lentivirus, or lentivirus expressing neurexin-2 $\beta$  without (-SS4) and with an insert in splice site 4 (+SS4), recorded as described for (C) and (D) (EPSCs: control: n = 11/2; Nr2 $\beta$  + SS4, n = 11/2; Nr2 $\beta$  -SS4, n = 11/2; IPSCs: control: n = 50/8; Nr2 $\beta$  + SS4, n = 51/8; Nr2 $\beta$  -SS4, n = 46/8).

(G) Representative images of neurons infected with control and neurexin-2 $\beta$  expressing lentiviruses stained for MAP2 and the vesicular GABA transporter vGAT.

(H) Quantitation of synapse area (left) and density (right) in the experiments described in (G) (control: n = 29/3; Nr2 $\beta$ : n = 40/3).

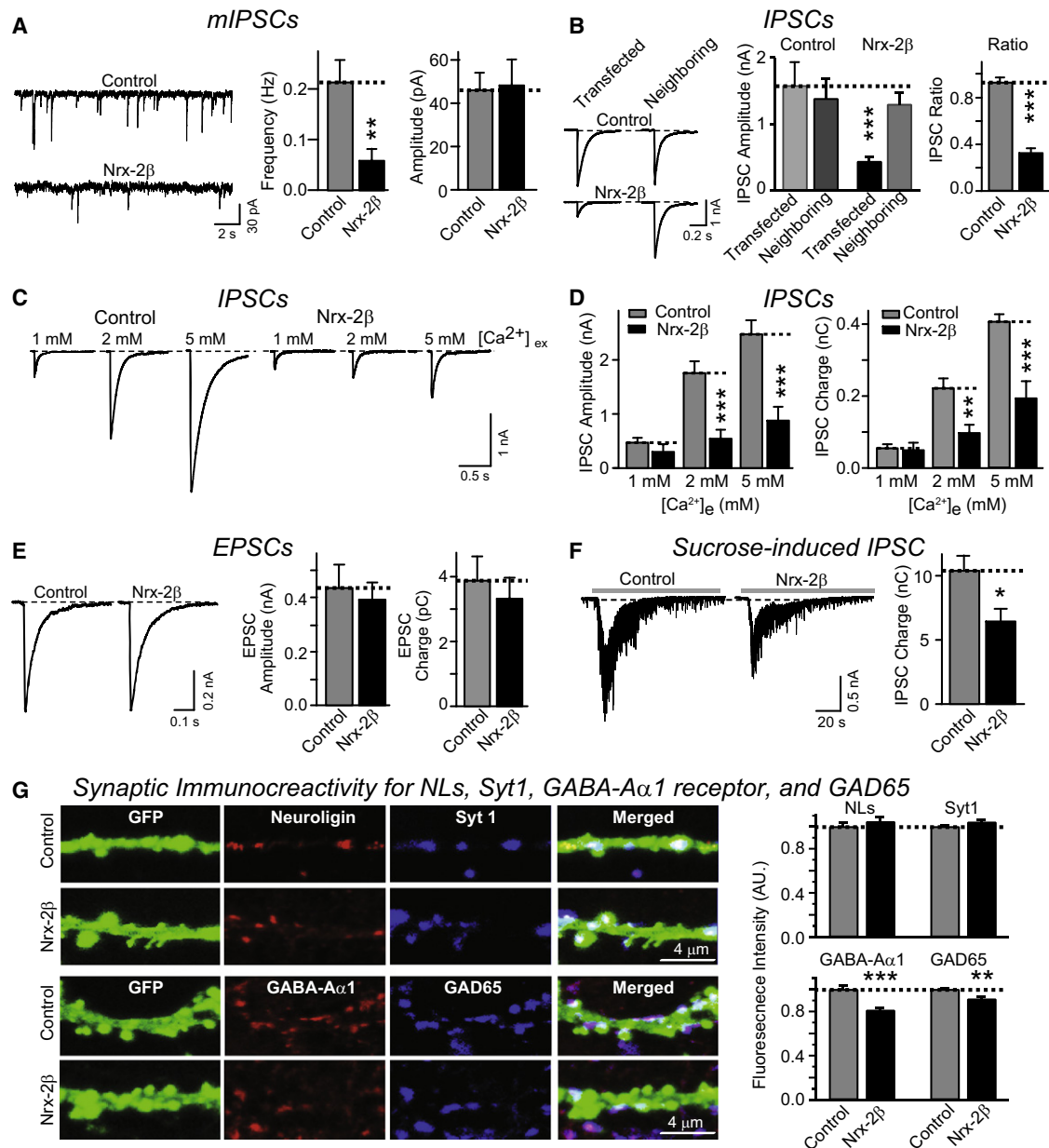
(I and J) Representative immunoblots (I) and summary graphs of protein levels (J) measured in lysates of cultured hippocampal neurons infected with control or Nr2 $\beta$ -expressing lentivirus. Protein levels were measured by quantitative immunoblotting with <sup>125</sup>I-labeled secondary antibodies and PhosphorImager detection (n = 3–5 cultures).

For all representative traces and images, scale bars apply to all panels in a set. All summary graphs show mean  $\pm$  SEM; statistical comparisons were made by Student's t test (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001). For additional data, see Figure S1.

To test whether all neurexins decrease GABAergic synaptic transmission, we tested neurexin-1 $\beta$ , -1 $\alpha$ , -2 $\beta$ , and -3 $\beta$ . All neurexins similarly impaired IPSCs (Figure 3A; for neurexin-1 $\alpha$ , see Figure 4B). Transfection of C-terminally truncated neurexin-2 $\beta^{\Delta$ ct lacking the cytoplasmic neurexin tail also decreased IPSCs, whereas deletion of the extracellular sequences of neurexin-1 $\beta$  abolished suppression of IPSCs, suggesting that postsynaptically expressed neurexin acts by an extracellular interaction (Figure 3B).

Does overexpressed neurexin block the maturation of new synapses, or impair the function of existing synapses? To

examine this question, we monitored the time course of neurexin-induced suppression of IPSCs. We transfected neurons with neurexin-2 $\beta$  or control vector at 10 days in vitro (DIV10), and measured the IPSC amplitudes 24–96 hr after transfection. Control-transfected neurons exhibited a continuous increase in IPSC amplitudes after DIV10, consistent with progressive maturation of synapses. In contrast, neurexin-2 $\beta$ -expressing neurons displayed no further increase in IPSC amplitudes after DIV10 (Figure 3C). Thus, neurexin-2 $\beta$  did not impair existing GABAergic synapses, but prevented the normal developmental increase in inhibitory synaptic strength. This observation cannot



**Figure 2. Postsynaptic Expression of Neurexin-2β Impairs Inhibitory Synaptic Strength**

All data are from cultured hippocampal neurons cotransfected with an EGFP expression plasmid and either an empty vector (control), or a vector encoding neurexin-2β (Nrx-2β).

(A) Representative traces (left) and summary graphs of the mean frequency (center) and amplitudes (right) of mIPSCs recorded in 1 μM TTX and 10 μM CNQX (control: n = 26/3; Nrx-2β: n = 24/3).

(B) Representative traces (left), mean amplitudes (center), and IPSC ratio (right) of IPSCs recorded from neighboring transfected and nontransfected neurons (control: n = 11 pairs/3 cultures; Nrx-2β: n = 12 pairs/3 cultures).

(C and D) Representative traces (C) and summary graphs of the amplitudes (D, left) and synaptic charge transfer (D, right) of IPSCs recorded in 1, 2, and 5 mM extracellular Ca<sup>2+</sup> (control: n = 17/3, 17/3 and 20/3 at 1, 2 and 5 mM extracellular Ca<sup>2+</sup>; Nrx-2β: n = 17/3, 18/3 and 23/3 at 1, 2, and 5 mM Ca<sup>2+</sup>).

(E) Representative traces (left), mean amplitudes (center), and mean charge transfer (right) of EPSCs recorded from control or neurexin-2β-transfected neurons in the presence of 10 μM picrotoxin (control: n = 28/4; Nrx-2β: n = 32/4).

(F) Representative traces (left) and mean charge transfer (right; integrated over 60 s) of IPSCs elicited by hypertonic sucrose (0.5 M for 30 s; control: n = 36/8; Nrx-2β: n = 42/8).

(G) Measurement of synaptic signals for neuroligins and GABA-A<sub>α</sub>1 receptors in neurons expressing neurexin-2β. Transfected neurons were stained with a pan-neuroligin antibody (Song et al., 1999) and synaptotagmin-1 (Syt1), or for GABA-A<sub>α</sub>1 receptors and GAD65. Panels show representative images (left) and quantifications of the staining intensity per synapse for all four markers (right).



be explained by an indirect effect of neurexins on synaptic activity, because chronic increases or decreases in synaptic activity (by incubating neuronal cultures with picrotoxin or with CNQX, APV, tetrodotoxin, or a combination thereof, respectively) caused no change in the suppression of IPSCs by neurexin-2 $\beta$  (Figure S3).

### Neurexins Act on GABAergic Responses Independent of Neuroligins

Neuroligin-2 is localized to inhibitory synapses, is essential for normal inhibitory synaptic strength in brain, and binds to all neurexins (Graf et al., 2004; Varoqueaux et al., 2004, 2006; Comoletti et al., 2006; Chubykin et al., 2007). Thus, neurexins may impair IPSCs by a selective sequestration of neuroligin-2.

To test this hypothesis, we coexpressed neurexin-1 $\beta$  or -1 $\alpha$  with neuroligin-1 or -2 in neurons, with the notion that because both neuroligins bind to neurexins, they would both similarly counteract the sequestration of endogenous neuroligin-2 by overexpressed neurexins. However, coexpression of neuroligin-1 had no effect on the suppression of IPSCs by neurexins. Neuroligin-2 alone enhanced IPSCs as described (Chubykin et al., 2007); coexpression of neuroligin-2 with neurexins canceled out the effects of each separate protein (Figures 4A and 4B). Moreover, we found that overexpression of neurexin-2 $\beta$  produced the same suppression of GABAergic responses in neurons from neuroligin-2 and -3 knockout (KO) mice (Figures 4C and 4D). Note that different from acute slices (Chubykin et al., 2007), neuroligin-2 KO neurons did not exhibit a decrease in IPSC amplitude in culture (Figure S4A). Furthermore, we found that a neurexin mutant (neurexin-2 $\beta$ <sup>D137A</sup>) that does not bind to neuroligins (Reissner et al., 2008; see also Figure 7 below) still effectively decreased IPSCs (Figure 4E). Finally, to explore whether neurexin binding to dystroglycan (Sugita et al., 2001) may decrease IPSCs, we examined the effect of dystroglycan transfection on IPSCs, either alone or in combination of neurexins. Dystroglycan had no effect either on IPSCs when transfected alone, or on the neurexin-induced suppression of IPSCs (Figure 4F).

Together, these data show that increasing neurexin expression does not impair inhibitory synaptic transmission by sequestering endogenous neuroligins. Given the primarily presynaptic localization of neurexins, the question arises whether transfected neurexins decrease inhibitory synaptic strength by a true postsynaptic action, or act in the postsynaptic neuron only after reaching synapses at the cell surface, and thus act by a quasi-presynaptic mechanism. Panneuronal loss-of-function experiments of neurexins cannot be used to address this question because loss of  $\alpha$ -neurexins alone already severely impairs neurotransmitter release, preempting any analysis of a postsynaptic effect (Missler et al., 2003). Thus, we addressed this question by testing whether a selective postsynaptic shRNA-dependent knockdown of all neurexins causes a phenotype opposite to the neurexin overexpression (Figures S4B–S4F), which would indicate a normally postsynaptic function of neu-

rexins in this regard, and whether expression of a neurexin mutant that is retained in the endoplasmic reticulum (ER) still suppresses inhibitory synaptic transmission (Figures 4G, 4H, and S4G–S4I).

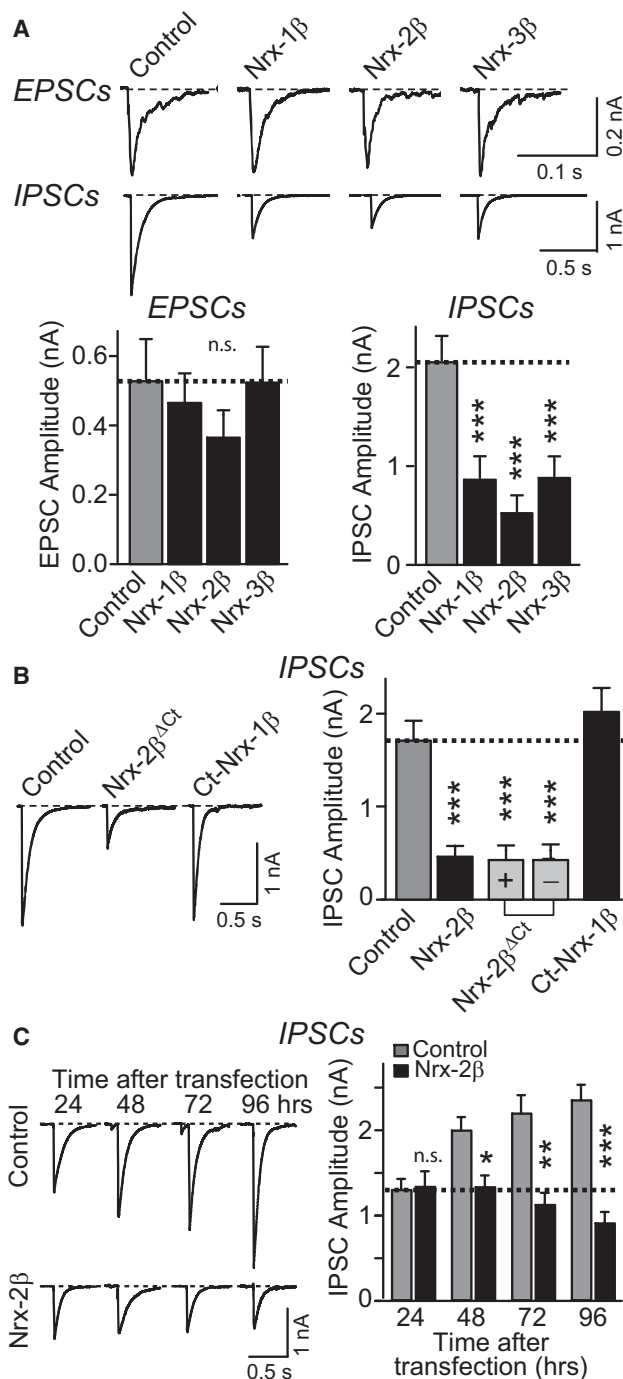
We developed shRNAs that efficiently knock down expression of all  $\alpha$ - and  $\beta$ -neurexins (Figure S4B). Expression of these shRNAs in transfected neurons, however, did not cause a change in inhibitory synaptic transmission, either in spontaneous or evoked synaptic responses (Figures S4C–S4F). Next, we expressed a soluble truncated neurexin-2 $\beta$  mutant that contains a C-terminal KDEL sequence (Nrx-2 $\beta$ <sup>KDEL</sup>). Nrx-2 $\beta$ <sup>KDEL</sup> still binds to neuroligins, but is retained in the ER, and had no effect on inhibitory synaptic transmission (Figures 4G, 4H, and S4G–S4I). Thus, neurexins have to be exported through the secretory pathway in order to act on GABAergic synapses, and transfected neurexins likely do not act in the postsynaptic neuron proper, but at synapses on the surface of the transfected neuron in a manner simulating a presynaptic action.

### Recombinant Neurexin-1 $\beta$ Added to the Medium Suppresses GABAergic Responses

If neurexins act on inhibitory synapses via an extracellular effect, recombinant neurexins added to the medium should simulate this action. To test this hypothesis, we incubated cultured neurons from DIV10 to DIV14 with a recombinant neurexin protein composed of the extracellular domains of neurexin-1 $\beta$  fused to the Fc domain of human immunoglobulin (IgNrx-1 $\beta$ ), using recombinant protein containing only the human immunoglobulin domain (IgC) as a control. IgNrx-1 $\beta$  specifically decreased the mIPSC frequency but not the mIPSC amplitude (Figure 5A), and suppressed evoked IPSCs but not evoked EPSCs; IgNrx-1 $\beta$  concentrations as low as 0.5  $\mu$ M were effective (Figures 5B, S5A, and S5B). Similar to transfected neurexins, IgNrx-1 $\beta$  caused a significant decrease in the readily releasable pool at GABAergic synapses (Figure 5C), but produced no detectable alteration in release probability as monitored with the paired-pulse ratio (Figure S5C). Moreover, protein measurements revealed that IgNrx-1 $\beta$  only produced a small change in proteins specific for GABAergic synapses without other major changes (Figure S5D). Thus, simple addition of extracellular neurexin-1 $\beta$  protein selectively suppresses GABAergic synapse function.

We next studied the time course of IgNrx-1 $\beta$  action on IPSCs. We either added 1  $\mu$ M IgNrx-1 $\beta$  at defined times between DIV10 and DIV14, and measured IPSCs at DIV14 (Figure 5D), or we added IgNrx-1 $\beta$  at DIV10, and measured IPSCs 24–96 hr afterwards (Figure 5E). In both types of experiments, addition of neurexin-1 $\beta$  prevented the normal developmental increase in IPSC size, without a significant effect on IPSCs already present at the time at which IgNrx-1 $\beta$  was added. These results, which agree with those obtained with transfected neurons (Figure 3C), indicate that neurexins do not inhibit existing GABAergic synapses in neurons, but prevent increases of GABAergic synaptic strength during the culture period.

For all representative data, scale bars apply to all panels in a set. All summary graphs show means  $\pm$  SEMs; statistical comparisons by Student's *t* test yielded: n.s. = nonsignificant, \**p* < 0.05; \*\**p* < 0.01, \*\*\**p* < 0.001. For additional data, see Figure S2.



**Figure 3. Extracellular Sequences of Neurexins Mediate Decrease of IPSCs**

Different neurexins were examined in transfected cultured hippocampal neurons as described for Figure 2.

(A) Representative traces (top) and mean amplitudes (bottom) of EPSCs and IPSCs recorded from neurons transfected with control vector or vectors encoding neurexin-1β (Nrx-1β), -2β (Nrx-2β), or -3β (Nrx-3β); EPSCs: control,  $n = 30/4$ ; Nrx-1β,  $n = 31/4$ ; Nrx-2β,  $n = 28/4$ ; Nrx-3β,  $n = 29/4$ . IPSCs: control,  $n = 42/4$ ; Nrx-1β,  $n = 23/3$ ; Nrx-2β,  $n = 48/4$ ; Nrx-3β,  $n = 21/3$ .

(B) Representative traces (left) and mean amplitudes of IPSCs (right) recorded from neurons transfected with control vector or vectors expressing wild-type

### Neurexins Interact with GABA<sub>A</sub> Receptors

Do neurexins interact with components of GABAergic synapses? To explore this possibility, we immobilized Ig-neurexin and Ig-control proteins, and performed affinity chromatography experiments with brain proteins solubilized in Triton X-100. Immunoblotting of bound proteins with a series of antibodies revealed specific binding of neuroligin-1 (as expected) and of GABA<sub>A</sub> receptors (both the  $\alpha 1$  and  $\beta 2/3$  subunits), but not of AMPA- or of NMDA-type glutamate receptors (Figures 6A, 6B, and S6A–S6C). Measurements of bound proteins relative to the flow-through showed that the GABA<sub>A</sub>1 receptor was retained quantitatively, with approximately half the efficiency of neuroligin-1 (Figure 6B). In contrast to neuroligins, binding of GABA<sub>A</sub>1 receptor to neurexin was  $\text{Ca}^{2+}$ -independent (Figure 6B), and a mutation in the neurexin  $\text{Ca}^{2+}$ -binding sequence that blocks neuroligin-1 binding had no effect on GABA<sub>A</sub>1-receptor binding (Figure 6A). These observations were fully confirmed using recombinant proteins expressed in transfected HEK293 cells (Figures S6D–S6G), suggesting that neurexins directly bind to GABA<sub>A</sub>1 receptors in a  $\text{Ca}^{2+}$ -independent manner.

Are endogenous GABA<sub>A</sub>1-receptor/neurexin complexes present in brain? We examined this question by affinity-purifying neurexins from brain homogenates with an immobilized glutathione S-transferase (GST) fusion protein containing the CASK PDZ-domain that avidly binds to the C-terminal cytoplasmic sequence of neurexins (Hata et al., 1996). GST-CASK captured both GABA<sub>A</sub>1 receptor and neuroligin-1 (Figures 6C and 6D). Quantitations of bound proteins suggested that similar amounts of the GABA<sub>A</sub>1 receptor and neuroligin-1 were bound by the CASK PDZ-domain (Figure 6D). This binding was not due to a direct interaction of the GABA<sub>A</sub>1 receptor with GST-CASK because GST-CASK could only affinity-purify the GABA<sub>A</sub>1 receptor from transfected HEK293 cells when neurexin-1β was coexpressed with the receptor (Figure S6H), demonstrating that GST-CASK pulls down a neurexin/ GABA<sub>A</sub>1-receptor complex.

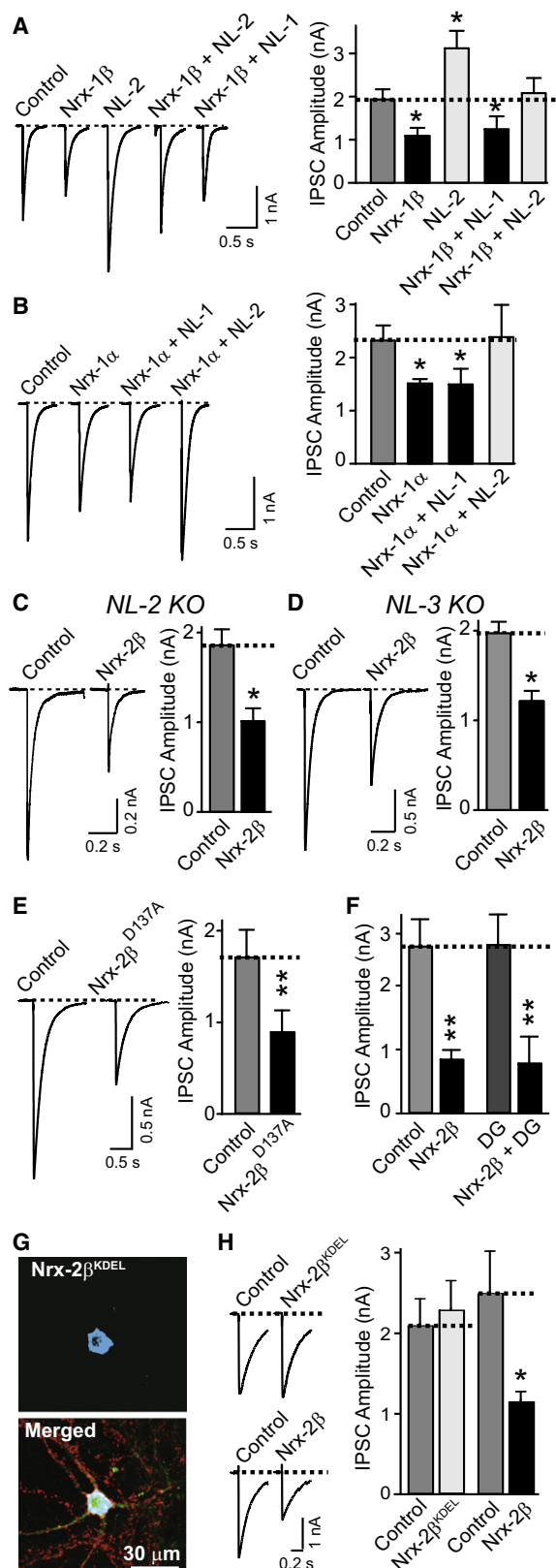
### Direct Extracellular Interaction of Neurexins and GABA<sub>A</sub> Receptors

The robust effects of neurexin on GABAergic synaptic transmission (Figures 1–5) could only be accounted for by their interaction if their extracellular domains bound to each other. Thus, we produced a recombinant GST-fusion protein of the extracellular domain of GABA<sub>A</sub>1 receptors, and tested its binding to purified Ig-neurexin. We observed specific binding when either neurexin

neurexin-2β (Nrx-2β), C-terminally truncated neurexin-2β (Nrx-2β<sup>ΔCt</sup>) composed of its extracellular sequences and transmembrane region with (+) and without (–) an insert in splice site 4, or N-terminally truncated neurexin-1β (Ct-Nrx-1β) composed of only its transmembrane region and cytoplasmic tail (control:  $n = 32/5$ ; Nrx-2β:  $n = 52/5$ ; Nrx-2β<sup>ΔCt</sup> without SS4:  $n = 32/5$ ; Nrx-2β<sup>ΔCt</sup> with SS4:  $n = 24/4$ ; Ct-Nrx-1β:  $n = 42/3$ ).

(C) Representative traces (left) and mean amplitudes (right) of IPSCs recorded from neurons transfected with control or neurexin-2β expressing vectors on DIV10, and analyzed at the indicated times after transfection (for all times tested: control,  $n = 5/3$ ; Nrx-2β,  $n = 15/3$ ).

For all representative traces, scale bars apply to all traces in a set. All summary graphs show mean  $\pm$  SEM; statistical comparisons by Student's *t* test yielded: n.s. = nonsignificant, \* $p < 0.05$ ; \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . For additional data, see Figure S3.



**Figure 4. Neurexins Impair IPSCs Independent of Neuroligins**

Hippocampal neurons transfected with the indicated combinations of expression vectors were analyzed as described for **Figure 2**.

(A) Representative traces (left) and mean amplitudes (right) of IPSCs recorded from neurons transfected with control vector, or vectors expressing only neurexin-1 $\beta$  (Nr $x$ -1 $\beta$ ), only neuroligin-2 (NL2), neurexin-1 $\beta$  together with neuroligin-2 (Nr $x$ -1 $\beta$  + NL2), or neurexin-1 $\beta$  with neuroligin-1 (Nr $x$ -1 $\beta$  + NL1; control: n = 42/5; Nr $x$ -1 $\beta$ : n = 55/5; Nr $x$ -1 $\beta$  + NL1: n = 26/4; Nr $x$ -1 $\beta$  + NL2: n = 25/4; NL2: n = 9/1).

(B) Representative traces (left) and mean amplitudes (right) of IPSCs recorded from neurons transfected with control vector, or vectors expressing only neurexin-1 $\alpha$  (Nr $x$ -1 $\alpha$ ), or together with neuroligin-2 (Nr $x$ -1 $\alpha$  + NL2), or only neuroligin-1 (Nr $x$ -1 $\alpha$  + NL1; control: n = 48/5; Nr $x$ -1 $\alpha$ : n = 38/5; Nr $x$ -1 $\alpha$  + NL1: n = 19/3; Nr $x$ -1 $\alpha$  + NL2: n = 16/3).

(C and D) Representative traces (left) and mean amplitudes (right) of IPSCs recorded from neurons transfected with control vector or neurexin-2 $\beta$  (Nr $x$ -2 $\beta$ ; C, control: n = 20/3; Nr $x$ -2 $\beta$ : n = 19/3; D, n = 9 pairs of neighboring nontransfected and transfected neurons/3 cultures).

(E) Representative traces (left) and mean amplitudes (right) of IPSCs recorded from neighboring nontransfected and transfected neurons expressing mutant neurexin-2 $\beta$  (Nr $x$ -2 $\beta$ <sup>D137A</sup>) in which a point mutation abolishes Ca<sup>2+</sup>- and neuroligin-binding to the neurexin-2 $\beta$  LNS-domain (control: n = 15 pairs/3 cultures).

(F) Mean amplitudes of IPSCs recorded from neurons transfected with control vector, or vectors expressing neurexin-2 $\beta$  alone (Nr $x$ -2 $\beta$ ), neurexin-2 $\beta$  together with dystroglycan (Nr $x$ -2 $\beta$  + DG), or dystroglycan alone (DG; control: n = 16/3; Nr $x$ -2 $\beta$ : n = 11/3; Nr $x$ -2 $\beta$  + DG: n = 16/3; DG: n = 12/2).

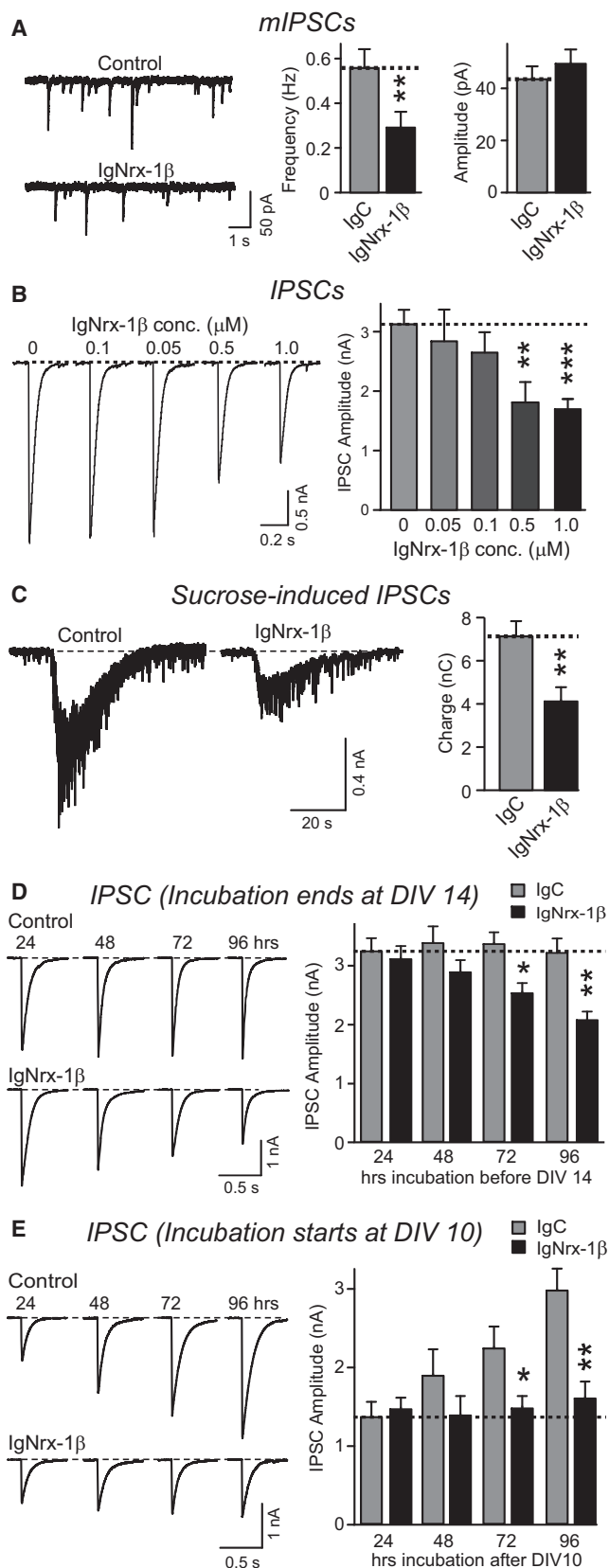
(G and H) Truncated neurexin-2 $\beta$  with a C-terminal KDEL sequence is retained in the ER (**G**) and unable to decrease IPSCs (**H**). Panel (**G**) depicts representative images of neurons demonstrating that neurexin-2 $\beta$ <sup>KDEL</sup> is retained in the ER (Nr $x$ -2 $\beta$ <sup>KDEL</sup>; visualized via a myc-epitope; top); colabeling for synapsin in the merged image reveals that the neuron nevertheless forms abundant synapses (Merged; bottom). Panel (**H**) (left, representative traces; right, summary graphs) shows that an ER-retained neurexin-2 $\beta$  mutant that still binds neuroligin (**Figure S4**) has no effect on IPSC amplitudes (Nr $x$ -2 $\beta$ <sup>KDEL</sup> transfection; control: n = 14/3; Nr $x$ -2 $\beta$ <sup>KDEL</sup>: n = 13/3; Nr $x$ -2 $\beta$  transfection; control: n = 8/3; Nr $x$ -2 $\beta$ : n = 8/3).

For all representative traces, scale bars apply to all traces in a set. Summary graphs show mean  $\pm$  SEM; statistical comparisons by Student's *t* test yielded: \**p* < 0.05; \*\**p* < 0.01. For additional data, including postsynaptic knockdowns of neurexins, see **Figure S4**.

or the GABA<sub>A</sub> $\alpha$ 1 receptor was pulled down (**Figures 7A** and **7B**). We next tested whether the GABA<sub>A</sub> $\alpha$ 1 receptor and neuroligin-1 could bind to neurexins simultaneously. Using recombinant proteins, we found that indeed, immobilized GABA<sub>A</sub> $\alpha$ 1 receptor captured not only neurexin-2 $\beta$ , but also neuroligin-1, demonstrating a tripartite interaction (**Figure 7C**). Finally, we examined the affinity of the neurexin-GABA<sub>A</sub> $\alpha$ 1 receptor interaction, using purified recombinant proteins that were not fused to GST or immunoglobulin (Ig) (**Figure 7D**). Using surface plasmon resonance measurements, we found a tight, specific, and saturable interaction between these two proteins with a micromolar affinity, confirming a direct binding reaction (**Figures 7E** and **7F**).

### Neurexins Inhibit GABA<sub>A</sub> $\alpha$ 1-Receptor Currents in Transfected HEK293 Cells

Does the interaction of neurexins with GABA<sub>A</sub> receptors directly decrease GABAergic currents? To test this question, we transfected neurexin-2 $\beta$  into nonneuronal HEK293 cells that stably express GABA<sub>A</sub> receptors on their surface (**Wong et al., 1994**).



**Figure 5. Recombinant Neurexin-1β in Medium Inhibits IPSCs**

All data are from cultured hippocampal neurons incubated with purified control Ig-fusion protein (IgC) or neurexin-1β Ig-fusion protein containing the extracellular domains of neurexin-1β (IgNrx-1β). Except where noted, neurons were treated for 96 hr at 37°C with 1 μM protein.

(A) Representative traces (left) and summary graphs of the frequency (center) and amplitudes (right) of mIPSCs monitored in 1 μM TTX and 10 μM CNQX (IgC: n = 19/3; IgNrx-1β: n = 19/3).

(B) Representative traces (left) and mean amplitudes (right) of evoked IPSCs recorded from neurons incubated with the indicated concentrations of IgNrx-1β (control: n = 17/3; 1 μM: n = 12/3; 500 nM: n = 13/3; 100 nM: n = 7/3; 50 nM: n = 7/3).

(C) Representative traces (left) and mean charge transfer (right) of IPSCs elicited by hypertonic sucrose (0.5 M for 30 s; IgC: n = 14/3; IgNrx-1β: n = 15/3).

(D) Representative traces (left) and mean amplitudes (right) of evoked IPSCs recorded from neurons treated with IgC or IgNrx-1β for the indicated times, with all incubations ending at DIV14 (24 hr: control, n = 19/3, IgNrx-1β: n = 14/3; 48 hr: control, n = 14/3, IgNrx-1β: n = 15/3; 72 hr: control, n = 19/3, IgNrx-1β: n = 19/3; 96 hr: control, n = 14/3, IgNrx-1β: n = 15/3).

(E) Representative traces (left) and mean amplitudes (right) of evoked IPSCs recorded from neurons treated with IgC or IgNrx-1β for the indicated times after the incubations were started at DIV10 (24 hr: control, n = 24/4, IgNrx-1β: n = 26/4; 48 hr: control, n = 18/3, IgNrx-1β: n = 18/3; 72 hr: control, n = 18/3, IgNrx-1β: n = 22/3; 96 hr: control, n = 18/3, IgNrx-1β: n = 18/3).

For all representative traces, scale bars apply to all traces in a set. All summary graphs show means ± SEMs; statistical comparisons by Student's t test yielded: n.s. = nonsignificant, \*p < 0.05; \*\*p < 0.01, \*\*\*p < 0.001. For additional data, see Figure S5.

Neurexin-2β robustly inhibited GABA-induced currents in the HEK293 cells (Figure 8A). Titration of the GABA-induced currents with different GABA concentrations revealed no change in the kinetics or dose-response curve, but a general suppression of the response (Figures 8B and S8). Moreover, incubation of HEK293 cells expressing GABA<sub>A</sub> receptors for 48 hr with purified neurexin protein (IgNrx-2β; 1 μM) also suppressed GABA currents (Figure 8C). Finally, transfected neurexin-2β decreased the levels of cellular GABA<sub>A</sub>-receptor protein (Figure 8D). Thus, neurexins appear to act directly on GABA<sub>A</sub> receptors.

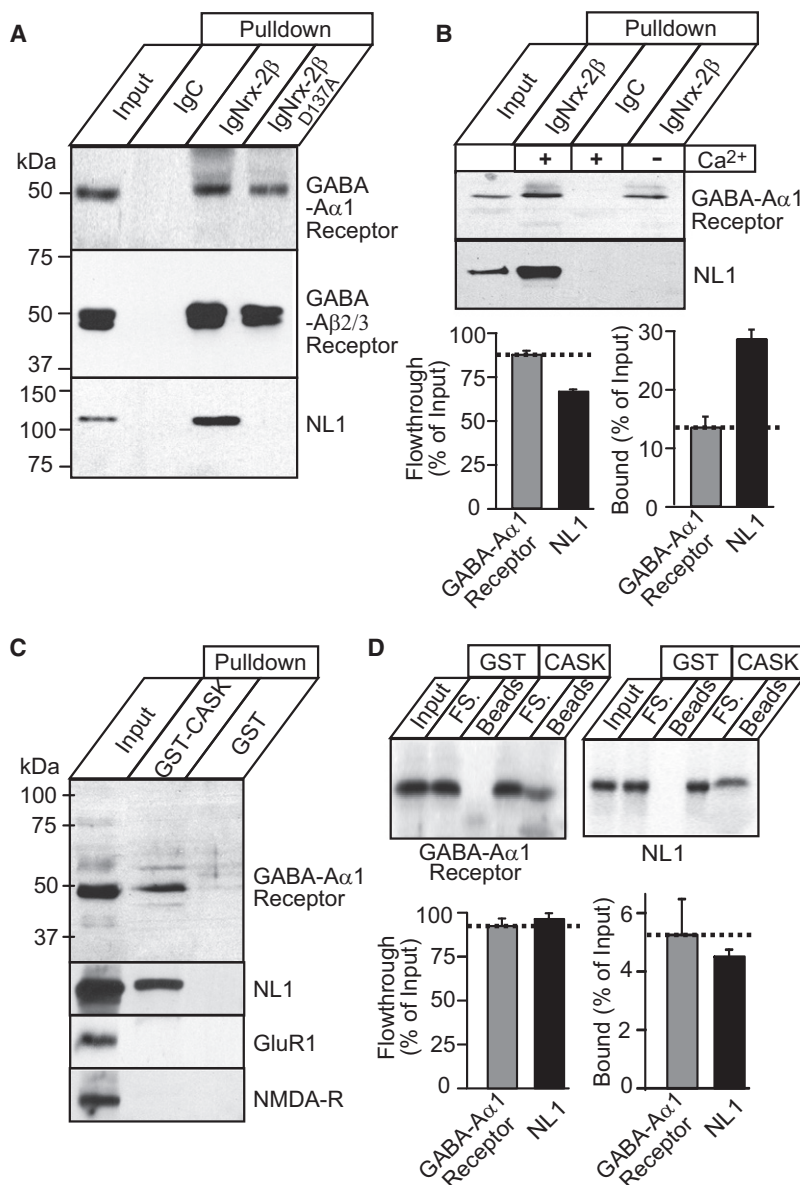
## DISCUSSION

Here we show that neurexins directly bind to the extracellular domains of GABA<sub>A</sub> receptors, and specifically inhibit postsynaptic GABA<sub>A</sub> receptors by a neuroligin-independent mechanism. We made six principal observations.

First, neurexins directly interact with GABA<sub>A</sub>α1 receptors, as shown by binding of recombinant IgNrx-2β to endogenous GABA<sub>A</sub>α1 receptors in brain and to transfected GABA<sub>A</sub>α1 receptors in HEK293 cells; coimmunoprecipitation of neurexin-1β with GABA<sub>A</sub>α1 receptors from transfected HEK293 cells; pulldown of endogenous brain neurexin/GABA<sub>A</sub>α1-receptor complexes and of recombinant neurexin/GABA<sub>A</sub>α1-receptor complexes with the CASK PDZ-domain; and measurements of the direct interaction of the purified extracellular domains of neurexin and GABA<sub>A</sub>α1 receptors (Figures 6, 7, S6, and S7).

Second, increasing neurexin expression decreases GABAergic but not glutamatergic synaptic transmission. This conclusion is based on lentiviral overexpression of neurexins (Figure 1), transfection of neurexins (Figures 2 and 3), and addition of recombinant neurexin to the medium (Figure 5). Neurexins





**Figure 6. Neurexins Form a Complex with GABA<sub>A</sub> Receptors**

All data shown are from affinity chromatography experiments using immobilized Ig- or GST-fusion proteins and Triton X-100 solubilized rat brain proteins. All summary graphs show mean  $\pm$  SEM. For additional raw data and controls, see Figure S6.

(A) Affinity purification of GABA<sub>A</sub> receptors on neurexin-2β. Immobilized IgC or Ig-fusion proteins of the extracellular sequences of wild-type neurexin-2β (IgNrx-2β) or of neurexin-2β with a Ca<sup>2+</sup>-binding site point mutation that blocks neuroligin-binding (IgNrx-2β<sup>D137A</sup>) were used as affinity matrices for rat brain proteins solubilized in Triton X-100. Bound proteins were analyzed by immunoblotting for GABA<sub>A</sub>α1- and GABA<sub>A</sub>β2/3 receptors and neuroligin-1. (B) Quantitative, Ca<sup>2+</sup>-independent binding of GABA<sub>A</sub>α1 receptor to IgNrx-2β. Solubilized rat brain proteins were bound to IgNrx-2β or IgC in the presence of 2.5 mM Ca<sup>2+</sup>, or of 0 mM Ca<sup>2+</sup> plus 5 mM EDTA. Bound proteins were analyzed by immunoblotting for GABA<sub>A</sub>α1 receptor and neuroligin-1 (NL1) with ECL detection (top), and additionally quantified for the Ca<sup>2+</sup>-containing experiments with <sup>125</sup>I-labeled secondary antibodies and PhosphorImager detection (bottom; mean  $\pm$  SEM, n = 8).

(C) Affinity chromatography of brain proteins on immobilized GST-CASK containing the CASK PDZ-domain (GST-CASK), or on GST alone. Bound proteins were analyzed by immunoblotting for GABA<sub>A</sub>α1 receptor, neuroligin-1 (NL1), GluR1, and NMDA receptor (NMDA-R).

(D) Quantitation of the binding of endogenous brain GABA<sub>A</sub>α1 receptor and neuroligin-1 (NL1) to immobilized GST-CASK or GST. Bound proteins were analyzed by immunoblotting with <sup>125</sup>I-labeled secondary antibodies and PhosphorImager detection; top panels show a representative blot, and bottom panels summary graphs (mean  $\pm$  SEM; n = 6). For additional raw data and controls, see Figure S6.

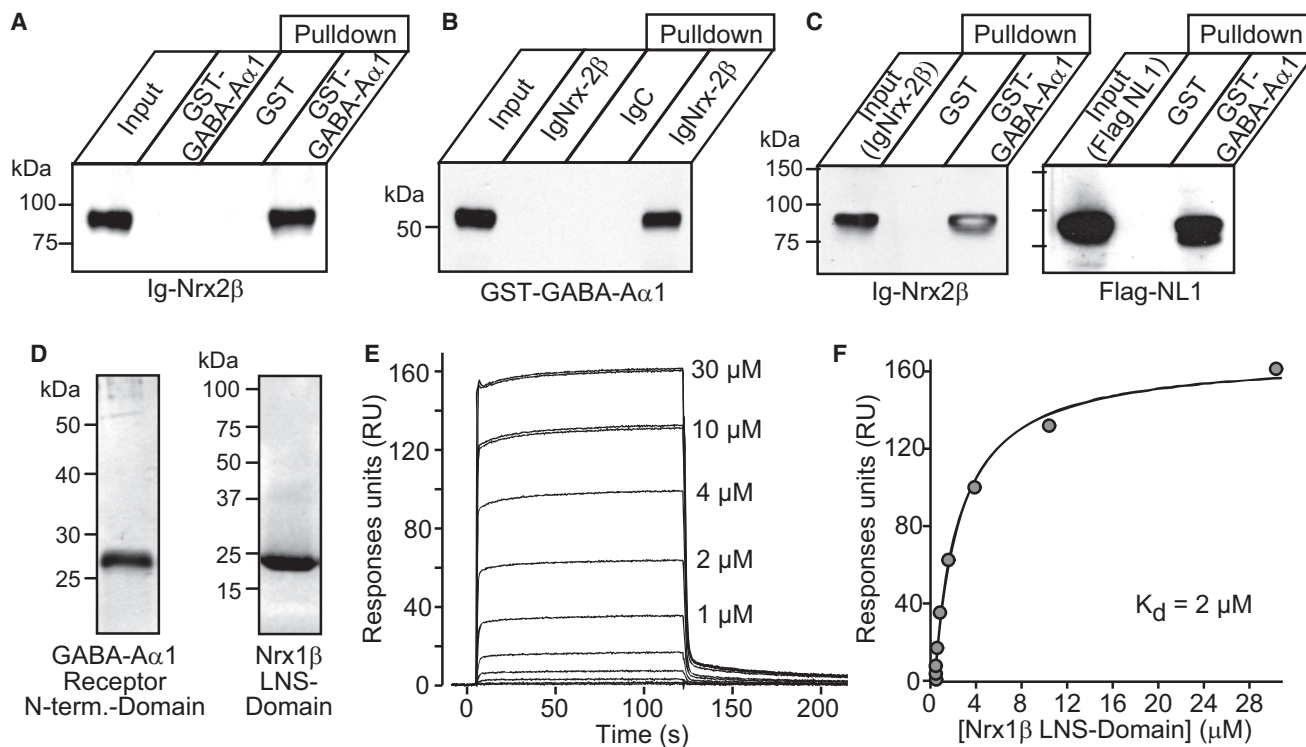
retained in the ER is inactive, and postsynaptic knockdown of neurexins has no effect (Figures 4 and S4).

Fourth, neurexins inhibit GABAergic responses independent of neuroligins. Cotransfection of neurexins with neuroligin-1 or neuroligin-2 did not ablate the inhibitory effect of neurexins on GABAergic synaptic transmission (Figures 4A and 4B); neurexin-2β still inhibited

have a large effect (>2-fold decrease in IPSCs in most assays) that is specific (no changes in EPSCs, no decreases in synapse numbers or major changes in neuronal protein composition; Figures 1–5 and S1–S5). Possibly most unexpected is the potency of recombinant neurexin that specifically suppressed IPSCs at only 0.5 μM (Figure 5). All neurexins tested had a similar effect that was independent of alternative splicing at site #4 (Figures 1–4).

Third, neurexins act via an extracellular mechanism on the neuronal surface. This was shown by the effectiveness of recombinant neurexin added to the culture medium (Figure 5), and of overexpressed neurexin-2β<sup>ΔCt</sup>, which lacks a cytoplasmic tail (Figure 3). In contrast, Ct-neurexin-1β, composed of only the common neurexin cytoplasmic tail and transmembrane region, was inactive (Figure 3). Moreover, a neurexin-2β mutant that is

GABAergic transmission in KO neurons lacking neuroligin-2, the only neuroligin specifically involved in inhibitory synaptic transmission (Chubykin et al., 2007), or neuroligin-3 (Figures 4C and 4D); recombinant neurexin added to cultured neurons had the same effect as transfected neurexins (Figure 5), which would not be expected if the overexpressed neurexins sequestered neuroligins in the neurons; a neurexin mutant that does not bind to neuroligins still bound to GABA<sub>A</sub> receptors, and still inhibited GABAergic synaptic transmission (Figures 4E and 6A); and a neurexin-2β mutant that is retained in the ER of neurons where it still binds to neuroligins did not decrease inhibitory synaptic strength (Figure 4). Note that the experiments with the various splice variants of neurexins and their mutants also rule out a participation of the recently discovered interaction of neurexins with LRRTMs (Ko et al., 2009b; de Wit et al., 2009).



**Figure 7. Neurexins Directly Bind to GABA<sub>A</sub>α1 Receptors**

All data shown analyze binding of purified recombinant proteins.

(A and B) Binding of purified GST or a GST-fusion protein of the N-terminal domain of human GABA<sub>A</sub>α1 receptor to Ig-control (IgC) or Ig-neurexin-2β fusion proteins (IgNrx-2β), analyzed either by immobilizing the GST-fusion proteins and measuring binding of IgNrx-2β (A), or immobilizing the Ig-fusion proteins and measuring binding of GST-GABA<sub>A</sub>α1 (B). Binding was visualized by immunoblotting as indicated.

(C) Binding of recombinant flag-tagged neuroligin-1 (NL1) to immobilized GST-GABA<sub>A</sub>α1 receptors by forming a tripartite complex with neurexin-2β. Immobilized GST or the GST-GABA<sub>A</sub>α1 fusion protein were incubated with both neurexin-2β and neuroligin-1, and bound proteins were analyzed by immunoblotting for neurexin-2β (left) or neuroligin-1 (right).

(D) SDS gels of purified proteins used for binding affinity measurements (left: silver-stained gel of the purified recombinant GABA<sub>A</sub>α1-receptor N-terminal domain; right: Coomassie-stained gel of the purified neurexin-1β LNS-domain).

(E) Surface-plasmon resonance sensorgrams showing the binding of neurexin-1β to the extracellular N-terminal domain of the GABA<sub>A</sub>α1 receptor. Each line corresponds to the indicated concentration of neurexin-1β injected onto the immobilized GABA<sub>A</sub>α1-receptor surface. The sensorgrams demonstrate a concentration-dependent association as expected for a bimolecular association.

(F) Surface-plasmon resonance quantitation of the binding of neurexin-1β to the N-terminal domain of the GABA<sub>A</sub>α1 receptor. The maximum steady-state binding of neurexin-1β to the GABA<sub>A</sub>α1 receptor is plotted as a function of the neurexin-1β concentration. Dissociation constants were calculated by fitting the curve to a single-site binding model. Each data point was measured twice, and both measurements are shown on the figure, but overlap with each other. The experiment was repeated with higher GABA<sub>A</sub>α1-receptor immobilization on the chip surface, and similar affinities were obtained (data not shown). For additional data, see Figure S7.

Fifth, neurexins likely act by binding directly to GABA<sub>A</sub>α1 receptors on the cell surface. This conclusion is inherent to the findings we made in neurons after addition of recombinant neurexin to the medium (Figure 5) and after transfection of various neurexin mutants (Figures 2–4), and further amplified by the finding that transfection of neurexin-2β into GABA<sub>A</sub>-receptor-expressing HEK293 cells or addition of IgNrx-2β to such cells suppressed GABA-induced currents in these non-neuronal cells (Figure 8).

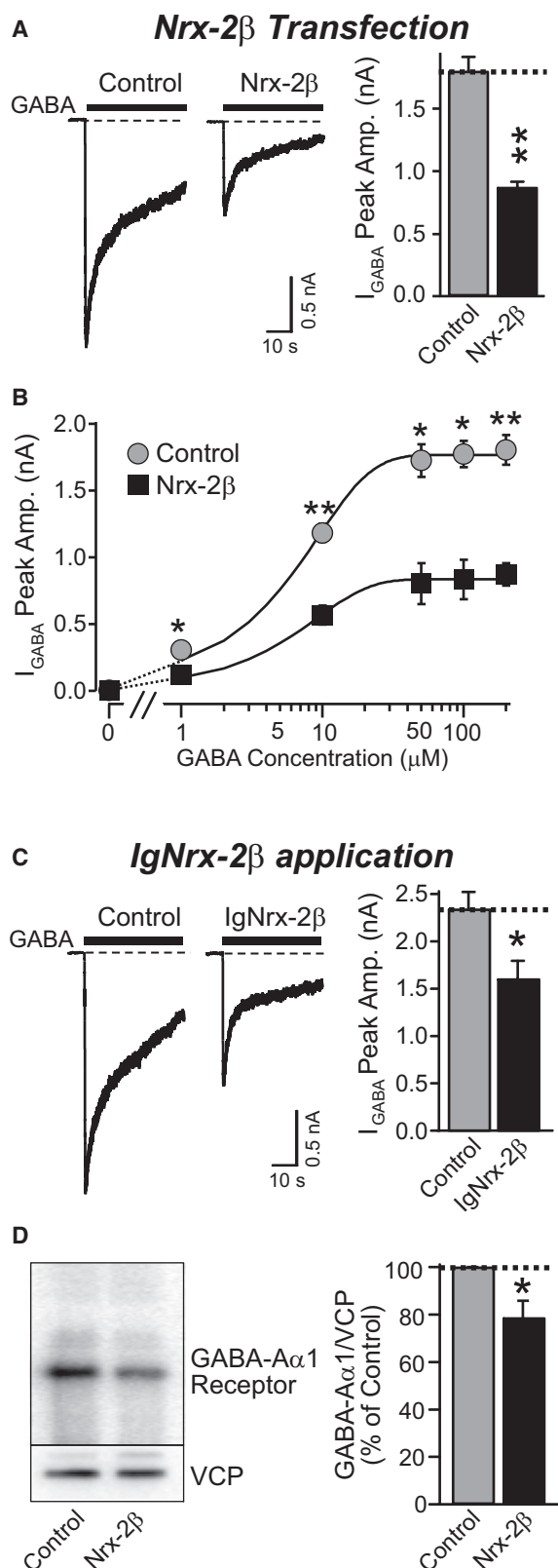
Sixth, finally, an excess of neurexins does not impair the functions of existing GABAergic sites—receptors and/or synapses—but prevents the developmental increase of GABAergic synaptic transmission in cultured neurons. Both for neurexin-transfected neurons and for untransfected neurons treated with recombinant neurexin, the neurexin “froze” the neuron in the state it

was at the point at which the treatment was started (Figures 3 and 5).

### Relation to Previous Studies

Our study appears to contradict nearly all previously published reports on neurexins, including many from our own laboratory. As described below, however, some of these contradictions may be more apparent than real.

We observed that excess neurexins selectively decrease inhibitory synapses, instead of promoting formation of excitatory synapses as suggested in studies using cocultures of neurons with transfected nonneuronal cells (Graf et al., 2004; Nam and Chen, 2005). However, the lack of an effect of neurexins on synapse formation is consistent with the finding that deletion of α-neurexins does not decrease the number of excitatory



**Figure 8. Neurexin-2 $\beta$  Impairs GABA<sub>A</sub> $\alpha$ 1 Receptor Function in Transfected HEK293 Cells**

(A) Representative traces (left) and peak amplitudes (right) of currents induced by application of 0.2 mM GABA to HEK293 cells stably expressing GABA<sub>A</sub> receptors ( $\alpha$ 1 $\beta$ 2 $\gamma$ 2, CRL-2029 from ATCC), and transfected with pCMV5 (control) or vector expressing neurexin-2 $\beta$  (control:  $n = 18/3$ ; Nrx-2 $\beta$ :  $n = 18/3$ ). (B) Plot of the current amplitude induced as described in A. as a function of the GABA concentration (control:  $n = 19/4$ ; Nrx-2 $\beta$ :  $n = 17/4$ ). For a scaled plot to document a lack of change in apparent GABA-affinity, see Figure S12.

(C) Representative traces (left) and peak amplitudes (right) of currents induced by application of 0.2 mM GABA to HEK293 cells stably expressing GABA<sub>A</sub> receptors that had been incubated for 48 hr with 1  $\mu$ M control IgG or IgNrx-2 $\beta$  (control:  $n = 15/3$ ; IgNrx-2 $\beta$ :  $n = 16/3$ ).

(D) Quantitation of the levels of GABA<sub>A</sub> $\alpha$ 1 receptor in HEK293 cells that stably express GABA<sub>A</sub> receptors, and were transfected with either an empty vector (control), or a vector encoding neurexin-2 $\beta$  (Nrx-2 $\beta$ ) ( $n = 13$  independent cultures).

For all representative traces, scale bars apply to all traces in a set. All summary graphs show mean  $\pm$  SEM; statistical comparisons by Student's  $t$  test yielded \* $p < 0.05$ ; \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . For additional data, see Figure S8.

synapses, and only moderately decreases the number of inhibitory synapses (Missler et al., 2003).

Moreover, we detected an apparently postsynaptic effect of neurexins, whereas neurexins are thought to function primarily presynaptically. However, our study shows that postsynaptically expressed neurexins act in the postsynaptic neuron only after transport to the cell surface, and thus may in fact mimic a presynaptic effect.

Furthermore, we propose that the observed effect of neurexins is independent of neuroligins, whereas most processes connected to neurexins can be accounted for by their binding to neuroligins. Indeed, because neurexins can be observed postsynaptically, they were speculated to be postsynaptic regulators of neuroligins (Taniguchi et al., 2007). However, there is no reason why neurexins should not bind to other proteins besides neuroligins (e.g., see Ko et al., 2009a; de Wit et al., 2009), and neuroligins also have neurexin-independent functions (Ko et al., 2009b).

Finally, our current results differ from those obtained with  $\alpha$ -neurexin KO mice (Missler et al., 2003; Kattenstroth et al., 2004; Zhang et al., 2005). Here, we observed a selective effect of neurexins on inhibitory synapses (i.e., it is not simple synapse damage) that is mediated by an extracellular cell-surface interaction of neurexins with postsynaptic neurons. In contrast, in the KO studies we found an essential function of  $\alpha$ -neurexins in organizing neurotransmitter release from all presynaptic nerve terminals. However, a specific change in inhibitory synaptic transmission would have been unobservable in  $\alpha$ -neurexin KO mice (Missler et al., 2003) because these mice exhibit an overwhelming impairment in neurotransmitter release, which would have occluded a separate facilitation of inhibitory synapses. Moreover, the continued expression of  $\beta$ -neurexins in these mice may have prevented manifestation of an inhibitory synapse phenotype. Thus, despite their differences, it seems likely that the two approaches revealed distinct parts of the overall picture of neurexin function.

### Physiological Significance

Our data suggest that neurexins decrease GABAergic synaptic transmission by direct binding to GABA<sub>A</sub> receptors on the cell surface at the synapse, and that neurexins act by impeding the

functional maturation of GABAergic synapses, not their initial formation. Thus, the effects of excess neurexins observed in our experiments may reflect a function of neurexins in regulating GABAergic synapse maturation. We propose that this newly observed activity is one facet of multiple actions of neurexins at synapses, and that presynaptic neurexins likely constitute master regulators of synapse maturation (or validation) via *trans*-synaptic interactions with an array of postsynaptic targets, which are only now slowly being discovered, as exemplified by the identification of LRRTMs as novel neurexin ligands (Ko et al., 2009a; de Wit et al., 2009). Teasing apart the physiological importance of the various interactions will require definition of neurexin mutations that specifically and selectively impair one interaction without affecting others, because the example of the  $\alpha$ -neurexin KO mice has already demonstrated that global ablation of neurexin expression is too blunt an instrument in order to define the full range of neurexin functions. However, the present observations uncovering a very robust action of neurexins on postsynaptic GABA<sub>A</sub> receptors extend and expand the emerging concept that neurexins function not in the initial establishment of synapses, but in shaping their properties, in their maturation. A key observation in this regard is the developmental effect of neurexins in the assays described here, which clearly shows that once synapses are formed, they are immune from the effect of overexpressed neurexins. Because synapses are likely continuously formed throughout adult life, this developmental action naturally continues beyond the actual development of the organism, and corroborates the notion of neurexins as key regulators of synaptic properties.

We propose that the effect of neurexins on postsynaptic GABA<sub>A</sub> receptors observed here is likely *trans*-synaptic. However, independent of whether the interaction of neurexins with GABA<sub>A</sub> receptors is postsynaptic or *trans*-synaptic, our findings may shed light on the role of neurexins in cognitive diseases such as autism, addiction, and schizophrenia (Szatmari et al., 2007; Kim et al., 2008; Yan et al., 2008; Marshall et al., 2008; Zahir et al., 2008; Kirov et al., 2008; Rujescu et al., 2009; Walsh et al., 2008; Hishimoto et al., 2007; Lachman et al., 2007; reviewed in Südhof, 2008). In these disorders, changes in inhibitory synaptic transmission are thought to play a major role. Specifically, alterations in the levels of GABA and GABA receptors (including GABA<sub>A</sub> $\alpha$ 1) in autistic patients, and mutations in genes expressing GABA receptor subunits (Collins et al., 2006; Fatemi et al., 2009) suggest that the GABAergic system may be centrally involved in autism. Additionally, extensive studies have indicated that mutations in GABA<sub>A</sub> $\alpha$ 1-receptor genes are associated with schizophrenia (Petryshen et al., 2005). In view of the fact that deletions of neurexin-1  $\alpha$  are also associated with autism and schizophrenia (Szatmari et al., 2007; Kim et al., 2008; Yan et al., 2008; Marshall et al., 2008; Zahir et al., 2008; Kirov et al., 2008; Rujescu et al., 2009; Walsh et al., 2008; Hishimoto et al., 2007; Lachman et al., 2007), and that neurexin-1 $\alpha$  KO mice exhibit traits resembling schizophrenia (Eherton et al., 2009), our observation that neurexins physically and functionally interact with GABA<sub>A</sub> receptors raises the tantalizing possibility that alterations in GABAergic synaptic transmission may contribute to a convergent mechanism for increasing the risk of autism and schizophrenia.

## EXPERIMENTAL PROCEDURES

### Construction of Expression Vectors

Various vectors were produced using standard molecular biology procedures (see Table S4 and Supplemental Experimental Procedures). The vector encoding YFP-tagged GABA<sub>A</sub> $\alpha$ 1-receptor was a gift from Dr. Robert Macdonald (Gallagher et al., 2005).

### Neuronal Cultures and Ca<sup>2+</sup>-Phosphate Transfections

Primary hippocampal neurons were cultured on poly-D-lysine-coated glass coverslips from 1- to 3-day-old mice (Maximov et al., 2007), and transfected with various vectors at DIV10 using a Ca<sup>2+</sup>-phosphate transfection method (see Supplemental Experimental Procedures). As visualized by the cotransfected CFP, 1%–2% of neurons were typically transfected.

### Lentiviral Infection

Lentiviruses were produced by transfecting HEK293 cells (CRL-11268, ATCC) with the respective pFUGW vectors and two helper plasmids (pVSvg and pCMV $\Delta$ 8.9). Viruses were harvested 48 hr after transfection by collecting the medium from transfected cells. Neurons were infected with 0.2–0.5 ml conditioned HEK293 cell medium for each 24 well of high-density neurons at DIV1–2, and the medium was exchanged to normal growth medium at DIV4, then kept until DIV13–DIV15 for biochemical and electrophysiological analyses. For all experiments, expression of exogenous proteins in neurons was confirmed by immunoblotting and GFP fluorescence.

Immunofluorescence analyses were performed as described previously (Chubykin et al., 2007), and analyzed blindly using the NIH ImageJ program. Electrophysiological recordings were performed as described elsewhere (Maximov and Südhof, 2005; Maximov et al., 2007) using a Multiclamp 700A amplifier (Molecular Devices).

Synaptic responses were evoked by 0.5 ms current injections (90–100  $\mu$ A) with an Isolated Pulse Stimulator (Model 2000, A-M Systems, Inc.) using a concentric bipolar electrode (FHC, CBAEC75) that was placed at a  $\sim$ 100  $\mu$ m distance from the patched neuron. The RRP was measured by perfusion of hypertonic sucrose (0.5 M) into the bath solution at 2 ml/min. Series resistance was compensated to 60%–70%, and recordings with series resistances of  $>$ 20 M $\Omega$  were rejected. Data were analyzed using Clampfit 9.02 (Molecular Devices) and Igor 4.0 (Wavemetrics).

### Recombinant Proteins

Ig-neurexins were produced in transfected COS cells and bacterial recombinant proteins were generated and purified as described previously (Boucard et al., 2005; Hata et al., 1996; Araç et al., 2007; see Supplemental Experimental Procedures).

### Protein Interaction Measurements Using Affinity Chromatography, Pulldowns, and Surface Plasmon Resonance Experiments

Affinity-chromatography experiments were performed with proteins solubilized in Triton X-100 from rat brain or from transfected HEK293 cells (Ichtchenko et al., 1995; see Supplemental Experimental Procedures). Surface plasmon resonance measurements were performed with the recombinant extracellular N-terminal regions of the neurexin-1 $\beta$  (Araç et al., 2007) and of the rat GABA<sub>A</sub> $\alpha$ 1 receptor (residues 28–251) essentially as described elsewhere (Araç et al., 2007; Garboczi et al., 1996; see Supplemental Experimental Procedures).

### Miscellaneous

SDS-PAGE and immunoblotting experiments, including quantitation with <sup>125</sup>I-labeled secondary antibodies followed by PhosphorImager detection (Molecular Dynamics) were performed as described elsewhere (Tabuchi et al., 2007). Most antibodies employed were previously characterized (Ichtchenko et al., 1995; Song et al., 1999; Sugita et al., 2001; Ushkaryov et al., 1992) or purchased (GABA<sub>A</sub> $\alpha$ 1 antibodies from Santa Cruz [sc-7348, dilution 1:200], or Alomone Labs [AGA-001, 1:200]). HEK293 cells stably expressing GABA<sub>A</sub> receptors were obtained from the ATCC (CRL-2029). All HEK293 and COS cell transfections were performed using FUGENE 6 (Roche Applied Science).



## Statistical Analyses

All data were generated in at least three independent experiments, and are shown as mean and standard error of the mean ( $\pm$ SEM). Statistical significance was determined by the Student's *t* test or the Kolmogorov-Smirnov test.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes eight figures, four tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.neuron.2010.04.008.

## ACKNOWLEDGMENTS

We would like to thank Dr. Robert L. Macdonald (Vanderbilt University) and Dr. Stephen Moss (Tufts University) for gifts of valuable reagents. This paper was supported by grants from the NIMH (MH52804 to T.C.S.) and the Simons Foundation (to T.C.S.), and fellowships from the Life Sciences Research Foundation (to D.A.) and the Human Frontier Science Program Organization (to J.K.).

Accepted: March 23, 2010

Published: May 12, 2010

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